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Direktor: Prof. Dr. Peter Deplazes

Arbeit unter Leitung von Prof. Dr. Ute Mackenstedt, Universität Hohenheim

Diagnosis of Sarcocystis spp. in cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*) in Northern Vietnam

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Christina Cäcilia Jehle

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Prof. Dr. Peter Deplazes, Referent

Prof. Dr. Bruno Gottstein, Korreferent

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Short communication

Diagnosis of *Sarcocystis* spp. in cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*) in Northern Vietnam

C. Jehle [a,*], A. Dinkel [a], A. Sander [a], M. Morent [a], T. Romig [a], P.V. Luc [b], T.V. De [b], V.V. Thai [b], U. Mackenstedt [a]

[a] Universität Hohenheim, Fachgebiet Parasitologie, Emil-Wolff-Str. 34, 70599 Stuttgart, Germany
[b] Department of Parasitology and Veterinary Hygiene, Hanoi Agricultural University, Hanoi, Viet Nam

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ABSTRACT

Our aim was to develop a method for species diagnosis and to obtain data on the prevalence of *Sarcocystis* infections in cattle and water buffalo in the Son La Province of Northern Vietnam. Meat samples of naturally infected animals were examined by light and electron microscopy as well as by molecular methods. A PCR of part of the 18S rDNA gene followed by RFLP analysis was modified to detect infections with different *Sarcocystis* spp. in cattle and water buffaloes slaughtered in the Son La Province. It showed to be an economical method to detect multiple infections with *Sarcocystis* spp. Sequence analysis of the PCR amplicons was performed with selected samples and the results were compared with published sequences. With these methods the following *Sarcocystis* spp. were identified in cattle: *Sarcocystis hirsuta*, *Sarcocystis cruzi* and *Sarcocystis hominis*.

Water buffaloes were infected with *Sarcocystis fusiformis*, *S. cruzi*, *S. hominis* and *S. hirsuta*. The results indicate that *Sarcocystis* spp. infecting cattle are also able to infect water buffaloes. So the validity of certain *Sarcocystis* spp. of water buffalo is discussed. Bovine lifestock in Northern Vietnam were commonly infected with *Sarcocystis* spp.

1. Introduction

Sarcocystis spp. are cyst-forming intracellular protozoan parasites with an obligate two host life cycle between predators as final hosts and their prey animals as intermediate hosts. *Sarcocystis* spp. are highly prevalent in livestock animals and are considered to be very host specific. Therefore, it is assumed that water buffaloes as well as cattle are solely infected with their own species. Cattle are mainly infected with *Sarcocystis cruzi*, *Sarcocystis hominis* and *Sarcocystis hirsuta* (Tenter, 1995; Dubey and Lindsay, 2006). Water buffaloes are intermediate hosts for *Sarcocystis fusiformis*, *Sarcocystis levinei* (*S. cruzi*-like species), *Sarcocystis dubeyi*, *Sarcocystis sinensis* (both *S. hominis*-like species) and *Sarcocystis buffalonis* (*S. hirsuta*-like species) (Dubey et al., 1989b; Huong et al., 1997a,b; Huong, 1999; Yang et al., 2001a,b). The *Sarcocystis* spp. can be differentiated due to their specific cyst wall structure (survey see Dubey et al., 1989a,b,c; Tenter, 1995) and by molecular methods as well as by sequence analysis (Tenter et al., 1994; Heckeroth and Tenter, 1999).

Few publications exist concerning the prevalences of *Sarcocystis* spp. in water buffaloes (Huong et al., 1997a,b; Huong, 1999) and similar data on prevalences in cattle from Northern Vietnam are lacking.

The aim of our project was to obtain data on the prevalence in these livestock animals.

We established a rapid method to distinguish between the different *Sarcocystis* spp. *S. cruzi*, *S. hominis* and *S. hirsuta* infecting cattle. PCR-RFLP analysis with different restriction endonucleases showed a unique fragment pattern for each of these species, so they can be clearly

* Corresponding author. Tel.: +49 711 45923575; fax: +49 711 45922276.
E-mail addresses: chrisjehle@gmx.de, jehle@landesjagdverband.de (C. Jehle).

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identified in a mixture of different *Sarcocystis* DNA. The results were confirmed by sequence analysis of the 18S rRNA gene.

To identify the different *Sarcocystis* spp. infecting water buffalo we based our method on the findings of Yang et al. (2001a,b, 2002) and Li et al. (2002). They compared *Sarcocystis* cysts (*S. cruzi*, *S. hominis* and *S. hirsuta*) from cattle and the corresponding cysts from water buffalo morphologically, by sequence analysis of the 18S rRNA gene and by PCR-RFLP. They combined the results and recognized that the *Sarcocystis* cysts from both cattle and water buffalo can be considered the same species (i.e. *S. cruzi*, *S. hominis* and *S. hirsuta*) and that water buffalo serves as their intermediate host in nature. Therefore, we used the same restriction endonucleases with samples of *Sarcocystis* DNA obtained from water buffalo and beneath the identification of *S. fusiformis* they showed identical fragment patterns with the samples obtained from cattle. These findings were supported by the results of sequence analysis of the 18S rRNA gene, showing an up to 100% identity with *Sarcocystis* spp. of cattle. Under these circumstances in the following the species names *S. cruzi*, *S. hominis* and *S. hirsuta* are used for *Sarcocystis* spp. infecting water buffalo, replacing the names *S. levinei*, *S. dubeyi* and *S. buffalonis*.

It was not our aim to identify *Sarcocystis* spp. using experimental infections but to find an easy method for species diagnosis via PCR-RFLP in naturally infected animals.

This project was part of the SFB (Sonderforschungsbereich) "Uplands Program – Research for sustainable land use and rural development in mountainous regions of Southeast Asia" funded by the Deutsche Forschungsgemeinschaft. Therefore, the research was restricted to rural areas in Northern Vietnam.

2. Materials and methods

The survey was done from October until December 2003 in 30 abattoirs in the town of Son La in the Son La Province in Northern Vietnam, where meat inspection is only performed sporadically and dogs and cats have often access to meat and offal disposals.

Meat inspection was done on 101 cattle and 30 water buffaloes, which originated from various small villages in Son La Province. From each animal tissue samples (each 30–50 g) from tongue, cervical muscle, oesophagus and diaphragm were collected during meat inspection. In the laboratory 0.5 mm sized pieces of the samples were squashed between two glass slides and inspected under a stereomicroscope (16× magnification) for microscopically visible *Sarcocystis* cysts. In total 541 samples of cattle and 208 samples of water buffaloes were inspected microscopically. If cysts were found in the sample, several 1 cm long pieces of tissue were fixed either in 70% ethanol or 10% neutral buffered formalin, the obtained macroscopically visible cysts were handled the same way. In total, 945 samples from cattle and 478 samples of water buffaloes were fixed for further studies. For light microscopy pieces of the formalin fixed samples were processed by standard histological techniques, sectioned at 7 μm, stained with Haematoxylin and Eosin and examined at 400–1000× magnification for *Sarcocystis* cysts.

For electron microscopy small pieces of tissue (max. 2 mm × 5 mm) containing many cysts and large single cysts were fixed in 2.5% glutaraldehyde in 0.1 M Soerensen buffer pH 7.4 and stored at 4 °C. The samples were post-fixed with 1% osmiumtetroxide and blockstained with 6% uranylacetate, dehydrated in graded ethanol and embedded in araldite. Ultrathin sections were stained with lead citrate and studied electron microscopically (LEO 912 AB).

DNA was isolated from all ethanol preserved samples positive for *Sarcocystis* according to Dinkel et al. (2004): 0.1–0.3 g of the samples were cut into small pieces and digested in the presence of 2 mg/ml proteinase K in 500 μl of 10 mM Tris–HCl (pH 7.5), 10 mM EDTA, 50 mM NaCl, 2% sodium dodecyl sulphate and 20 mM dithiothreitol. DNA was extracted using phenol–chloroform extraction and subsequent ethanol precipitation (Sambrook et al., 1989). After drying, DNA was suspended in 100 μl nuclease-free water and its concentration measured photometrically.

For PCR the target sequence chosen for amplification was part of the mitochondrial 18S rRNA gene. Those variable regions have shown to be suitable genetic markers for distinguishing *Sarcocystis* spp. (Yang et al., 2002). Primers were used according to Li et al. (2002), the forward primer 18S9L (5′ GGA TAA CCT GGT AAT TCT ATG 3′) and the reverse primer 18S1H (5′ GGC AAA TGC TTT CGC AGT AG 3′) amplifying a fragment of approx. 900 bp. The used primer combination is *Sarcocystis* genus-specific and does not amplify host DNA (Li et al., 2002).

PCR was performed in a total volume of 100 μl using 400 ng DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, each desoxynucleoside triphosphate at a concentration of 200 μM, 60 pmol of each primer and 2.5 units Ampli-Taq Polymerase (Applied Biosystems) as follows: 94 °C for 3 min, followed by 40 cycles of 94 °C for 40 s, 56 °C for 60 s, 72 °C for 80 s and finally 72 °C for 5 min.

Amplification products as well as a negative control were separated on a 1.5% agarose gel stained with ethidium bromide (TBE-buffer, 55 V, 45 min).

For PCR-RFLP analysis the available sequences of the 18S rDNA gene of the different *Sarcocystis* spp. were extracted from GeneBank. The sequences were compared and restriction sites were identified using the Harvard program (http://pga.mgh.harvard.edu/web_apps/web_map/start) in order to differentiate between different *Sarcocystis* spp. as tissue samples may have contained cysts of more than one *Sarcocystis* sp. Therefore, banding patterns generated by PCR-RFLP include the individual fragment patterns each of which is characteristic for one species indicating multiple infections. The accession numbers, the chosen restriction endonucleases for RFLP and the species specific fragment sizes are listed in Table 1. Amplified PCR products were digested separately with restriction enzymes. A total of 50 μl reaction mixture was used containing 10–20 μl PCR product, 10 units restriction enzyme and 5 μl appropriate buffer. The restriction mixture was incubated for 16 h at 37 °C (Dra1, Ssp1) or 55 °C (Fok1, Bsl1). Enzymes were inactivated for 20 min at

Table 1
Species specific fragment sizes of RFLP digestion with the enzymes Fok1, Dra1, Bsl1 and Ssp1.

	Fok1	Dra1	Bsl1	Ssp1
S. cruzi	535, 321[1–4]	444, 412[1–4]	513, 343[1–4]	0[1–4]
S. hominis	0[1–5]	0[1–5]	303, 238, 213, 98[1–5]	0[1,2,3,5] or 637, 233[4]
S. sinensis	0[1–4]	0[1] or 442, 414[2,3,4]	298, 236, 213, 109[1,2] or 511, 236, 109[3,4]	0[1–4]
S. hirsute	517, 207, 102, 81[1–5]	0[1–5]	525, 241, 141[1–5]	647, 260[1–5]
S. buffalonis	512, 207, 81, 77	0	525, 242, 110	n.p.*, 617, 260
S. fusiformis	0[2,3] or 639, 246[1]	768, 99[1–3]	532, 335[1–3]	n.p.*, 613, 266[1–3]

Small numbers mark the corresponding gene sequences with accession numbers.
n.p.*: no RFLP was performed due to the comparable fragment sizes of the *Sarcocystis* spp.
S. cruzi (1: AF017120; 2: AF176932; 3: AF176933; 4: AF176934).
S. hominis (1: AF006470; 2: AF006471; 3: AF176942; 4: AF176943; 5: AF176944).
S. sinensis (1: AF176929; 2: AF176930; 3: AF176931; 4: AF266954).
S. hirsuta (1: AF017122; 2: AF176938; 3: AF176940; 4: AF176941; 5: AF006469).
S. buffalonis AF017121.
S. fusiformis (1: U03071; 2: AF176926; 3: AF176927).

65 °C (Dra1 and Ssp1) and at 80 °C (Fok1 and Bsl1) according to the manufacturer's recommendations (Fermentas, USA). The obtained restriction fragments were separated on 2% agarose gels stained with ethidium bromide (TBE-buffer, 55 V, 45 min). A DNA size marker (Gene Ruler™, 700–25 bp Low Range DNA-Ladder, Fermentas) was used to identify the fragment sizes.

As a reference method the PCR products (approx. 900 bp) of the 18S rDNA gene of 10 selected samples obtained from cattle and twelve samples from water buffaloes (which contained DNA of only one *Sarcocystis* sp. as determined by PCR-RFLP) were sequenced. PCR was performed as described above and cycle sequencing was done after purification of the PCR products with QiaquickTM columns on the Gene Amp 2700 (Applied Biosystems) with the ABI prism big dye terminator cycle sequencing ready reaction kit (Applied Biosystems) using the corresponding PCR primers 18S9L (forward) and 18S1H (reverse). Depending on the quality of the obtained sequences an additional reaction was performed using the forward primer 2Lforw (5′ > GGA TAA ACC GTG GTA ATT CTA TG < 3′) (Yang et al., 2001a,b) as well as a newly designed reverse sequencing primer SEQREV (5′ CTC TGA CAG TTA AAT ACG AAT GCC C 3′). 25 cycles with the following conditions were performed: denaturation for 10 s at 96 °C and annealing for 4 min at 60 °C. Electrophoresis was carried out on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). The nucleotide sequence analysis was done using the National Centre for Biotechnology Information BLAST programs (Version Blastn 2.2.10) and databases (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2).

3. Results

To differentiate between the individual *Sarcocystis* spp., light and electron microscopical studies as well as molecular studies were performed. *Sarcocystis* spp. were highly prevalent in cattle and water buffalo: 63 cattle (63%, CI 53–73%) and 27 water buffaloes (90%, CI 73–98%) were infected with *Sarcocystis* spp. No significant differences between the prevalences of male and female animals were found in either host species.

3.1. Microscopy

In the light microscopy of 25 samples obtained from cattle the thin-walled cysts of *S. cruzi* could be easily differentiated from the thick-walled cysts that characterize infections with either *S. hominis* or *S. hirsuta*. Two different cyst wall structures could be differentiated by means of electron microscopy. One cyst wall structure had hair like villar protrusions which are characteristic for *S. cruzi*.

Additionally in one sample a cyst was identified with a smooth wall of 800 nm. It revealed stubby protrusions of about 400 nm in diameter. Between these protrusions the cyst wall revealed branched invaginations (Fig. 1). This cyst wall structure resembles the wall structure of a *Sarcocystis* sp. described by Odening et al. (1996) in a dwarf zebu kept in a zoo of Berlin. The cyst wall is similar to the wall structure of *Sarcocystis sibirica* (resp. *Sarcocystis gracilis*) infecting roe deer with dogs as possible final hosts (Dubey et al., 1989a).

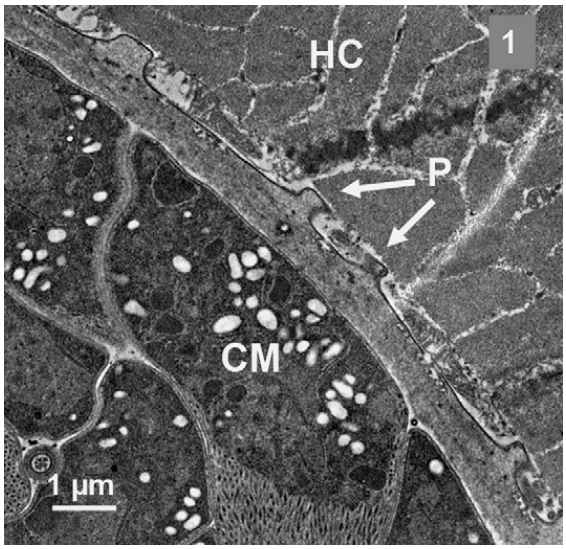
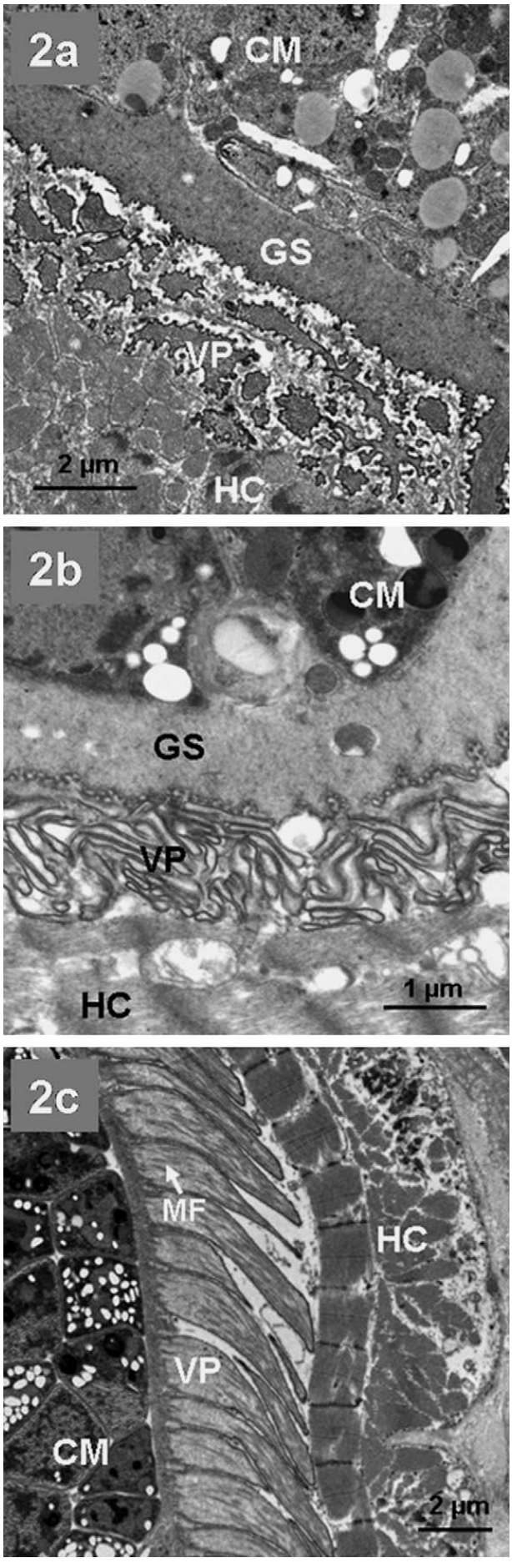


Fig. 1. Transmission electron micrographs of *Sarcocystis* sp. found in cattle. CM = cyst merozoite; HC = host cell; P = stubby protrusions.



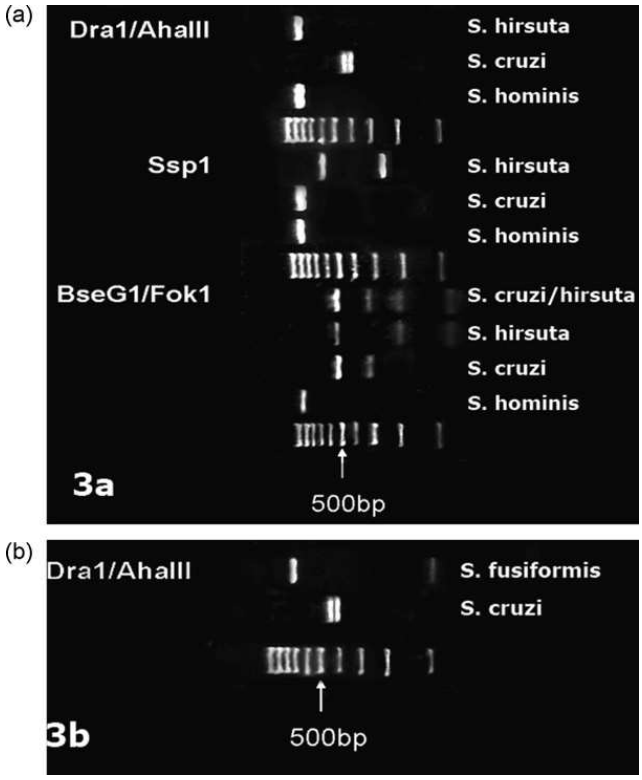


Fig. 3. (a) Exemplary PCR-RFLP analysis of DNA isolated from infected cattle. Summary of the species specific fragment sizes in Table 1. (b) PCR-RFLP analysis of DNA isolated from infected water buffalo showing the restriction pattern of *S. fusiformis* and *S. cruzi*.

The light microscopical studies of 16 samples from water buffaloes revealed thin- as well as thick-walled cysts indicating different *Sarcocystis* infections. Thin-walled cysts are characteristic for *S. cruzi* or *S. fusiformis* whereas *S. hominis*, *S. sinensis* and *S. hirsuta* show thick-walled cysts.

As mentioned before only highly infected intermediate hosts have been studied by means of electron microscopy. In 27 examined samples from water buffaloes the following cyst wall structures were identified: Cysts of *S. fusiformis* with the cauliflower-like protrusions (Fig. 2a) and *S. cruzi* cysts (Fig. 2b) characterized by hair like villar protrusions. Additionally cysts with villar protrusions bend at a 45° were identified, which may indicate an infection with *S. hominis* or *S. sinensis* (Fig. 2c).

3.2. PCR-RFLP

From 63 cattle positive for *Sarcocystis* cysts after meat inspection, DNA was isolated from 483 different pieces of the infected organs (oesophagus, diaphragm, tongue and cervical muscle) followed by PCR and RFLP. Fig. 3a shows an exemplary RFLP digestion of several samples from cattle tissue containing DNA of *S. hirsuta*, *S. cruzi* and *S. hominis* as well as a combination of these species. The fragment sizes are listed in Table 1. The combined results of all PCR-RFLP

Fig. 2. Transmission electron micrographs of (a) *S. fusiformis*; (b) *S. cruzi*; (c) *S. sinensis* or *S. hominis*. Note the 45° bend villar protrusions. HC = host cell; CM = cyst merozoite; GS = ground substance; VP = villar protrusions; MF = microfilaments.

Table 2
Prevalence of *Sarcocystis* spp. of cattle including multiple infections.

Sarcocystis spp.	*S. cruzi* *S. hominis* *S. hirsuta*	*S. cruzi* *S. hominis*	*S. cruzi* *S. hirsuta*	*S. hominis* *S. hirsuta*	*S. cruzi*	*S. hominis*
Number of infected cattle	23	23	2	3	7	5

Table 3
Prevalence of *Sarcocystis* spp. of water buffalo including multiple infections.

Sarcocystis spp.	*S. fusiformis* *S. cruzi* *S. hominis* or *S. sinensis* *S. hirsuta*	*S. fusiformis* *S. cruzi* *S. hominis* or *S. sinensis*	*S. cruzi* *S. hominis* or *S. sinensis*	*S. fusiformis* *S. cruzi*	*S. fusiformis*	*S. cruzi*
Number of infected water buffaloes	1	10	3	8	4	1

are summarized in Table 2. The results indicate that multiple infections with different *Sarcocystis* spp. are common in Northern Vietnam.

27 water buffaloes were positive for *Sarcocystis* spp. after meat inspection. DNA was isolated from 77 samples of muscle, tongue, diaphragm and oesophagus tissue and PCR was performed. The results of the different restriction analyses are summarized in Table 3.

The PCR-RFLP analysis showed a unique fragment pattern for *S. cruzi* and *S. fusiformis* after restriction with the restriction endonucleases Fok1 (*S. cruzi*) and Dra1 (*S. fusiformis*), so these species can be clearly identified in a mixture of different *Sarcocystis* DNA. Fig. 3b shows an exemplary RFLP digestion of two samples containing DNA of *S. fusiformis* and *S. cruzi*.

Several sequences of the 18S rRNA gene of *S. hominis*, *S. sinensis* and *S. hirsuta* are published (accession numbers listed in Table 1). Sequences of different strains of *S. sinensis* were published by Yang et al. (2001b). These sequences showed either restriction patterns similar to *S. hominis* (Fok1, Dra1, Bsl1, Ssp1), to *S. cruzi* (Dra1, Ssp1) or to *S. hirsuta* (Dra1, Bsl1). No restriction enzyme could be identified which produced unique fragment patterns for the different sequences of *S. sinensis* and allowed a differentiation from *S. hominis*. As a result *S. sinensis* cannot be identified from a DNA mixture of different *Sarcocystis* spp. *S. hirsuta* can be distinguished from *S. hominis*, *S. cruzi* and *S. fusiformis* by the restriction enzymes Fok1, Bsl1 and Ssp1. In summary the following endonucleases were selected to differentiate between the different *Sarcocystis* spp. (Table 1): the restriction enzymes Fok1, Dra1 and Bsl1 for *Sarcocystis* spp. infecting water buffalo; Fok1, Dra1 and Ssp1 for bovine species.

3.3. Sequencing

Ten different DNA isolates of *S. cruzi*, *S. hominis* and *S. hirsuta* obtained from cattle and identified by PCR-RFLP were amplified and sequenced, using the forward primer 2Lforw and two different reverse primers (SEQREV and 18S1H). The sequences were compared with published sequences of these species using the NCBI BLAST program. The homology between the obtained *S. cruzi* sequences and the sequence AF017120 was 99%. The sequences of *S. hirsuta* were 98% resp. 99% identical with the published sequences (AF017122, AF006469). Sequencing of *S. hominis* did not provide the complete sequence because elongation ceased with both reverse primers. Therefore, no overlapping sequence with the forward primer could be obtained. The homologies between the forward sequences and the published sequences AF006470 and AF006471 were 96–98%.

The sequence analysis revealed that the sequences of the 18S rRNA gene of *Sarcocystis* cysts from water buffalo were in five cases nearly identical with the corresponding sequences of *S. cruzi* (AF176932: 99% identity, AF176935: 100%) and in two cases with *S. hominis* (AF176945, AF176944: 99%), i.e. *Sarcocystis* spp. of cattle. In case of one sequence obtained from a *S. hominis*-like cyst the BLAST sequence results showed a difference in 10 bases (98%) towards the sequence of *S. sinensis* (AF176930) as well as to the sequence of *S. hominis* (AF176945).

The obtained sequences of *S. fusiformis* (three samples) matched the already published sequences (AF176926: 99%, AF176927: 100%) of this *Sarcocystis* sp. Of all analysed samples only two samples from one water buffalo showed the characteristic RFLP pattern of *S. hirsuta* respective *S. buffalonis*. Sequence analysis revealed that the obtained sequences showed 99% identity with the published sequences of *S. buffalonis* (AF017121) as well as with the sequences of *S. hirsuta* (AF176940, AF176941) differing each in only one to three bases.

4. Discussion

Data on the prevalence of *Sarcocystis* spp. in Vietnam are scarce or even lacking. Huong (1999) studied water buffaloes in different areas of Vietnam and described the highest prevalences (average 89%) in the northern part of Vietnam. The results of the present study revealed that 90% of the water buffaloes were infected which supports the results of Huong (1999), Huong and Uggla (1999) and Huong et al. (1997a,b).

To our knowledge nothing is known about the prevalence of *Sarcocystis* spp. in cattle in Northern Vietnam. Of the slaughtered cattle 63% harboured the

parasite. Three *Sarcocystis* spp. have been unequivocally identified in cattle: *S. cruzi*, *S. hominis* and *S. hirsuta* (Dubey et al., 1989a; Dubey and Lindsay, 2006). Additionally the electron microscopical studies presented in this paper revealed another cyst wall structure which is not comparable with the *Sarcocystis* sp. mentioned above. This cyst wall structure resembles the wall structure of a *Sarcocystis* sp. described by Odening et al. (1996) in a dwarf zebu kept in a zoo of Berlin. The cyst wall is similar to the wall structure of *S. sibirica* (resp. *S. gracilis*) infecting roe deer with dogs as possible final hosts (Dubey et al., 1989a). Yang et al. (2001b) described a similar cyst wall structure in cattle, however further studies are necessary to characterize this *Sarcocystis* sp. in detail.

All *Sarcocystis* spp. found in lifestock are considered to be host specific. So Huong et al. (1997a) proposed the name *S. buffalonis* for a *S. hirsuta*-like sarcocyst in water buffalo. The same authors redescribed the *Sarcocystis* sp. *S. levinei* in water buffalo (1997b), resembling the structure of *S. cruzi*. Huong and Uggla (1999) proposed the name *S. dubeyi* for a thick-walled microscopic sarcocyst occurring in water buffalo. The cyst wall ultrastructure is similar to that of the bovine species *S. hominis*, with villar protrusions sometimes bending laterally at a 45° angle. According to this description, Yang et al. (2001a) proposed that *S. dubeyi* might be the same species as *S. sinensis*, also having finger-like villar protrusions bent at a 45° angle to the surface of the cyst wall.

In our study cysts with this ultrastructure were found in samples from water buffalo (Fig. 2c). Due to the controversial descriptions mentioned above, they might be cysts of *S. hominis* as well as of *S. sinensis*. Cysts with hair like villar protrusions, characteristic for *S. cruzi* (Fig. 2b) were also found in samples from water buffalo. This diagnosis was affirmed by the results of PCR-RFLP and sequencing.

Yang et al. (2001a,b) started to identify *Sarcocystis* spp. in water buffalo using the 18S rRNA gene sequence and questioned the existence of some of the *Sarcocystis* spp. described in water buffalo. In their studies, single cysts were extracted from fresh muscle tissue. After a preliminary species diagnosis, DNA was isolated from each cyst followed by PCR analysis. They aligned 18S rRNA gene sequences of *S. hominis*-like, *S. hirsuta*-like, *S. cruzi*-like and *S. sinensis* cysts from water buffalo with the sequences of the morphologically corresponding cysts from cattle. The average sequence divergence between similar organisms from different intermediate hosts are all in the range of the same *Sarcocystis* spp. ($P > 0.05$), but in contrast, lower than that from different *Sarcocystis* spp. ($P < 0.05$). In a phylogenetic tree based on the 18S rRNA gene sequences the morphologically similar species from cattle and water buffalo formed a distinct cluster as one species. Dahlgren et al. (2008) made a phylogenetic tree for the Sarcocystidae based on ssu rRNA gene sequences. The phylogeny indicates a close relationship between *S. hominis* and *S. sinensis* as well as between *S. hirsuta* and *S. buffalonis*.

Yang et al. (2002) and Li et al. (2002) used a PCR-RFLP analysis for characterization of *Sarcocystis* spp. in cattle and water buffalo. The PCR-RFLP analysis revealed that *S. buffalonis* is identical with *S. hirsuta*. The *S. hominis*-like, *S. hirsuta*-like, *S. cruzi*-like and *S. sinensis* cysts of water buffalo show the same restriction pattern as the corresponding species of cattle. These results suggest that the morphologically similar species from two different intermediate hosts (cattle and water buffalo) should be considered as one species. This presumption is supported by the successful experimental infections of water buffaloes with *S. cruzi* by Xiao et al. (1991) and with *S. hominis* by Chen et al. (2003), indicating that some of the *Sarcocystis* spp. are able to infect more than one intermediate host and are therefore not as host specific as predicted.

The aim of our study was to obtain data on the prevalence of *Sarcocystis* infections in cattle and water buffalo in the Son La Province of Northern Vietnam and to develop a method for species diagnosis in naturally infected animals. DNA was isolated from tissue samples containing cysts of different *Sarcocystis* spp. The DNA mixture was amplified with PCR and RFLP analysis was performed. An isolation of the cysts before performing PCR was not necessary. According to the combination of the species specific restriction band patterns, multiple infections with up to four *Sarcocystis* spp. could be detected. This method worked with muscle samples from cattle and water buffalo. To our knowledge it is the first time that multiple *Sarcocystis* infections could be identified using one piece of host muscle containing cysts of different *Sarcocystis* spp. instead of isolated *Sarcocystis* cysts. PCR-RFLP showed to be an economical method to identify different *Sarcocystis* spp. infecting one intermediate host. However PCR-RFLP does not allow a differentiation of *S. sinensis* from *S. hominis*. Due to the variety of the published sequences of *S. sinensis* no restriction enzyme could be detected that produces a unique fragment band pattern for *S. sinensis*. The results of the sequence analysis showed a difference in 10 bases towards the sequence of *S. sinensis* (AF176930) as well as to the sequence of *S. hominis* (AF176945).

The results of the PCR-RFLP analysis and the gene sequences presented in this paper revealed a high degree of homology between the *Sarcocystis* spp. of cattle and water buffalo although only partial sequences were compared and additional data are needed. Together with the above mentioned results of the different publications the host specificity of some *Sarcocystis* spp. is questioned. It is therefore presumed that the *Sarcocystis* spp. *S. levinei*, *S. dubeyi* and *S. buffalonis* described in water buffalo are the same species as *S. cruzi*, *S. hominis* and *S. hirsuta* infecting cattle. For confirmation transmission and cross-transmission experiments should be performed.

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Lebenslauf

Name	Christina Cäcilia Jehle
Geburtsdatum	31. Juli 1975
Geburtsort	Stuttgart
Nationalität	deutsch

1982 – 1986	Grundschule in Leonberg
1986 – 1995	Johannes Kepler Gymnasium Leonberg
1995	Abitur
1995 – 1996	Studium der Kommunikationswissenschaft an der Universität Hohenheim, Deutschland
1997 – 2003	Studium der Veterinärmedizin an der Ludwig-Maximilian-Universität München, Deutschland
2000 – 2001	Studium am Tierspital in Zürich
11.09.2003	Approbation als Tierärztin an der Ludwig-Maximilian-Universität München, Deutschland
2003 – 2005	Wissenschaftliche Mitarbeiterin im SFB 564 der DFG an der Universität Hohenheim, Deutschland
2005 – 2006	Assistenztierärztin in der „Tierärztlichen Klinik für Kleintiere Elmer–Kornberg–Schanen“ in Trier, Deutschland
seit 2006	Referentin für Wildbrethygiene, Wildkrankheiten, Jagdhundewesen und Jägerprüfung beim Landesjagdverband Baden-Württemberg e.V., Stuttgart, Deutschland

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